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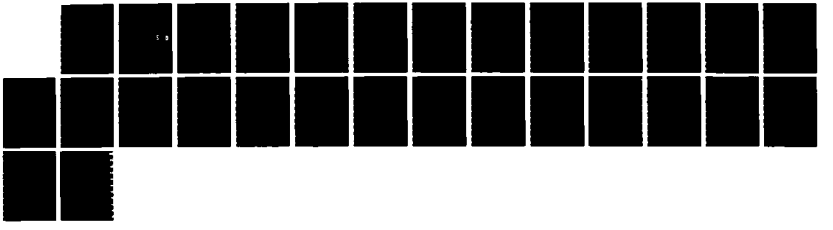

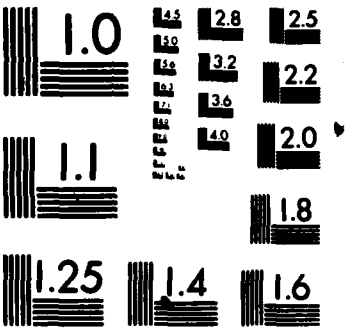

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Oligonucleotide Fingerprinting of Dengue Virus RNA to Support Ongoing Epidemiological Investigations

ANNUAL REPORT

BY

GEORGE E. FOX

SEPTEMBER 13, 1984

SUPPORTED BY

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MARYLAND 21702

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An information system for analyzing newly determined dengue virus fingerprints in the context of an on line data base of previously determined fingerprints is being developed and tested. We are digitizing the original autoradiograms and then analysing the resulting digital image to produce files of features that allow rapid reconstruction of a good fascimile of the original spot pattern on a suitable graphics device. These feature files allow sufficently efficient storage of the information that we will

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be able to maintain a large data base on line in a dedicated computer. We also have developed preliminary software to register the images so that rapid judgements can be made about the number of spots that have equivalent mobilities on any two images. Software is also availble to use data for many such binary comparisons to perform cluster analysis to determine hierarchical relationships among the various viral strains. Considerable progress has been made in most areas of the project . The next step is to integrate the various peices into a unified system. This we hope to accomplish in the forthcoming year.

All problems have not yet been overcome. Improvements and modifications are needed in almost all important software areas. Nevertheless we feel that we are far enough along with the development work that we can begin the production phase of the contract. Although considerable software development is still needed our most serious problems at present relate to hardware limitations. The processing time required by the segmentation program is far too extensive to allow us to follow an efficient production schedule. On the VAX computer which will support the data base is a shared system. As the data base grows we will face increasing difficulties in obtaining enough memory to maintain it unline. In the immediate time period we are suffering significant delays due to competition for system resources with instructional users. This problem has become increasing severe due to changes in the University's philosophy on computing.

Our major recommendation is that we attempt to perform production work in the coming year and thereby begin establishing an integrated software system. In order to accomplish this we believe it is essential to upgrade the processor on the minicomputer that is used for the segmentation work and to begin examining alternative options to use of the campus VAX system for storing the data base. We also recommend that some input regarding useful features be obtained from test users during this second year. In this regard installation of a suitable graphics terminal at the CDC laboratory which is preparing the fingerprints should be considered.

FORWARD

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PROBLEM STATEMENT:

The assigned task is to develop an automated system for analyzing newly determined dengue virus fingerprints in the context of an on line library of previously existing fingerprints. It is intended that such an analysis will allow a rapid identification of previously examined isolates that most closely resemble any new isolate. It is also hoped that correlations of various features of the fingerprints can be made with epidemiologically significant properties of the various isolates.

BACKGROUND:

Dengue viruses, the etiologic agents of "classical" dengue fever and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are distributed over tropical and subtropical regions of all five continents (1). In urban settings the viruses are transmitted to man principally by Aedes aegypti mosquitoes and the resourcefulness of this vector in adapting to tropical urban environments is believed to be a major factor contributing to a dramatic increase during recent years in DHS/DSS (2).

Half of the world's population resides in areas where dengue virus is endemic and 350 million people live in areas where there have been DHF/DSS epidemics during the last decade. In Thailand in 1977, DHF/DSS was the second leading cause for hospitalization of children and the leading cause of death due to communicable diseases at any age. Dengue viruses play a prominent role in pediatric morbidity and mortality in several major tropical population areas of the world (2).

Although the statistical data accumulated during the past two decades suggest that dengue viruses are an increasing public health problem, many basic

questions concerning their epidemiology and pathogenesis remain unanswered. Why do certain populations contract severe forms of dengue disease and others only "classical" dengue fever? The "second-infection" hypothesis for DHF/DSS (i.e., sequential infections with heterologous dengue serotypes), initially proposed by Halstead et al. (3), is supported by a variety of epidemiological studies (cf. Halstead for a review). In vivo studies probing the mechanisms involved in this hypothesis have given rise to the immune enhancement theory (4). Alternative explanations for the DHF/DSS syndrome have been put forward by Hammon (5) and Rosen (6). Essentially, these workers argue for virus-specific virulence factors as the source of different responses by different populations to dengue virus epidemics (i.e. "classical" vs. DHF/DSS disease). However, few data describing genetic markers which differentiate viruses within a given serotype are available, and the choice or reconcilation between these two alternatives must await such data.

Although serological methods, haemagglutination inhibition (HI), complement fixation (CF) and plaque reduction neutralization tests (PRNt), subdivide dengue viruses into four serotypes (7), they are not sufficiently sensitive to distinguish among members of a given serotype. With a method of sufficient sensitivity, it might be possible to differentiate a virus isolate which has the potential for causing DHF/DSS from one which does not have this capability.

The method of oligonucleotide fingerprint mapping yields distinct patterns for different members of a given virus family (8-12). The oligonucleotide maps from strains of La Crosse virus (LAC) with no detectable difference in serological characteristics range from (a) identity to (b) minor but clearly

distinguishable differences to (c) fingerprints with no apparent relationship to one another (10). Thus oligonucleotide fingerprints are a very sensitive method for measuring relationships between viruses which are indistinguishable by other criteria. For example, the method has ben used to develop a dendrogram of the genetic relationships between various isolates of LaCrosse viruses from the western U.S. (11).

These studies suggest that the method has sufficient sensitivity to quantitatively measure small differences between dengue virus isolates from around the world. An initial study of four prototype strains of dengue virus, representing each of the serotypes, showed that less than 10% of their unique oligonucleotides were identical (13). These same investigators then proceeded to show that oligonucleotide maps from dengue viruses within a given serological type (DEN1) showed varying degrees of relatedness as a function of how close in time or geographic location they had been isolated.

Isolates from within a given geographical area (Africa, the Caribbean and Pacific/S.E. Area) demonstrated 45% to 100% homology in their unique oligonucleotides (i.e., ≥ 10 bases long) while only 20% to 30% of the unique oligonucleotides were identical in viruses from <u>different</u> geographical areas. Comparison of the patterns from a DEN1 virus, first detected in the Caribbean in 1977, with DEN1 isolates from other regions of the world demonstrated the highest homology (50% to 60%) between the Caribbean isolate and isolates from Sri Lanka and Nigeria suggesting that the new Caribbean virus came originally from one or the other of these areas (13). More recently a detailed study of Dengue 2 strains (14) showed that geographically isolated and epidemiologically

unrelated viruses had very distinct fingerprints whereas strains that were related shared 80-95% of their large oligonucleotides.

The work on DEN1 (13), DEN2 (14) and LAC viruses (10) has established that the method can distinguish arbovirus isolates from different geographic areas. It is therefore of great value in epidemiological studies on dengue viruses. However, actual sequencing of the individual oligonucleotides on a particular RNA fingerprint is tedious and time consuming. The usual approach has thus been for individual investigators to visually compare the spatial location of spots between various fingerprints. This becomes increasingly time consuming as the number of binary comparisons increases and is subject to inevitable variations caused by different interpretations made by individual scientists. The entire procedure would be greatly facilitated by the availability of a computer-based hardware and software system which could rapidly analyze the data in an accurate uniform manner. In addition, this system could bring all the fingerprint data together into a single site that would be accessible to all interested investigators in the field. Development of such a computer-based system is the major goal of the work being conducted for this contract.

COLLABORATIVE ASPECTS OF THE WORK:

The work is being performed by two groups on separate contracts. A group under the direction of Dr. Dennis Trent at the Centers for Disease Control Laboratories in Fort Collins, Colorado is responsible for preparation of viral RNA and the fingerprints. The University of Houston group is responsible for preparation of the software and maintenance of the data base once it is established. This latter work is described in this report.

GENERAL APPROACH:

The intent is to reduce each fingerprint to a set of feature files that can be readily stored in an on line data base. These feature files will contain sufficient information to allow in most instances the comparison of any new isolate of dengue fever virus with previously examined isolates witnout resorting to reexamination of the fingerprint of the earlier isolate. The information contained in these feature files should allow construction of a display for any known isolate that bears close resemblance to the original fingerprint. These feature files must be sufficiently simplistic that they do not require prohibitive amounts of storage. Once this data base is established it is necessary to develop software to compare individual fingerprints to one another and to analyze trends in the data base as a whole.

Our approach is to use modern image processing technology to establish the requisite data base. The first step is to digitize the original x-ray films produced by the fingerprinting procedure. This is currently done by scanning the image with a vidicom camera equipped with either a Cannon TV-14 25mm lens or a Cannon TV-16 13mm wide angle lens. The camera is interfaced to a Spatial Data Eyecom III image analysis system which gives a video output to 16 bits with 256 gray levels ranging from 0 (white) to 255 (black). The resulting image consists of a 640 x 480 pixel array stored on a DEC PDP 11/23 computer.

The digitized image is subsequently segmented to separate the oligonucleotide spots from the background. The current system for doing this examines the numerical second derivative of the image in order to find core spots which are subsequently propagated (15). The segmented image is the key to obtaining a manageable data base and is thus the crucial software development step.

From the segmented image a feature file is to be obtained which allows rapid construction of a facsimile of the original image. At present we believe that three features will accomplish this. These are the set of pixels that define the boundary of the spot in the segmentation, the total optical density found in the spot, and the location of any optical density maximums. Subsequent intercomparison of any two images is accomplished by a registration program which utilizes a second or third order bi-linear transformation to intercompare images. This program requires the choice of appropriate spots which are clearly equivalent to define the geometric warping or in the case of quite dissimilar fingerprints the inclusion of external markers.

The registered images can be examined visually to determine how many spots have the same mobility and in this way a quantitative measure of the degree of similarity between all combinations of isolates can be calculated. These individual measures of similarity are used as input data to cluster analysis programs which construct dendrograms that show the relationships between the individual isolates. These same dendrograms also will provide key information to efficient arrangement of the strains in the data base.

RESULTS and DISCUSSION of RESULTS:

During the first year we have largely developed most of the components of the system described above. It remains to solve residual problems, establish the data base and to generally integrate the software together. In this section we will review progress in several key areas in more detail.

A. Segmentation:

Segmentation of the images is the process by which the computer decides which pixels are actually included in spots and which are not. A successful

segmentation algorithm is essential to automate the location of spots, to define those pixels which should be included in the calculation of key parameters such as total spot intensity and spot center, and to define a boundary for use in the data base in order to allow display of a good facsimile of the original image. The success of the project rests to a large extent on obtaining a good segmentation and for this reason this aspect has received the most attention during the first year.

The thing that makes the segmentation problem difficult is that despite their appearance to the human eye, actual spots blend quite gradually into the background and do not have a well defined edge. In order to circumvent this it is usual to attempt to enhance the edge of the spot in some way. We first attempted to do this with an algorithm using a numerical first derivative for which we had existing source code. This approach was successful in finding many spots but generally failed because intensity levels on the images are not uniform. This is the result of single spots which contain either several copies of the same oligomer or several oligomers with essentially identical composition.

After several attempts to improve this algorithm we decided that the best strategy would be to adapt an algorithm which has been successfully used in the analysis of protein electrophoresis gels (15). Because this algorithm was only available in computer language (SAIL-Stanford Artificial Intelligence Language) that cannot be run on either the VAX or PDP-11/23 it was necessary to laboriously convert it to FORTRAN. This conversion was done on a VAX 11/780 as well as the PDP 11/23 system on which the actual processing will be conducted and operational code currently exists for both machines. The VAX version has

been very useful in debugging but will not be used in the actual processing due to limited access. The invested time has proven fruitful in that we now have an essentially operational segmentation algorithm.

The most serious difficulty now confronting us with the segmentation is computation time. In order to set up the data base we will have to process the existing backlog of fingerprints from the earlier studies as well as the new fingerprints from the current study. In addition as refinements are added to our package it will inevitably be necessary to reprocess all or some of the images again. While we are doing this catching up we will also have to process the many new images being produced by the CDC group which is now in the production phase. Our PDP 11/23 currently requires approximately four hours to fully process a single full resolution image (a considerable improvement over the 16 hours that was required when the algorithm was first implemented). We have been unable to find any way of further accelerating the calculations and thus have explored other options. In particular we have purchased hardware to allow remote communication with the machine so that the segmentation algorithm can be run at night. We have also explored a possible upgrade of the system to the newer PDP 11/73 configuration. Since calculational speed is the primary limiting factor rather than input/output operations we can expect a 50-70% improvement if we are able to make this upgrade.

Other minor difficulties also remain with the segmentation. We still have problems at full resolution due to local fluctuations in the second derivative. These can be alleviated by improving the quality of the histogram obtained during segmentation and by modifying the neighborhood included in the second

derivative calculation from the current 5 x 5 to 7 x 7. The algorithm also is occasionally missing pieces of spots or generating extra spots. In part these problems can be resolved by more proficient use of the built-in selection criteria. The problems that remain can likely be traced to a less than optimal weighting of the 5 x 5 neighborhood used in calculating the second derivative. In the long run these difficulties will be resolved. In the short run we intend to temporarily finesse them by adding some simple editing functions to allow minor operator directed revisions.

B. Image Rectification:

It is important to facilitate image comparison. In order to do this one would like to eliminate the inevitable variations that occur from one run to the next so that images could be overlapped and spots of identical mobility identified. To effect such an inter-comparison of images a group of register points common to both images is selected. The register points of one image are mapped onto the plane formed by the register points of the second image. This amounts to fixing the plane of one image and then warping the plane of the other so that both sets of register points overlap relative to an observor orthogonal to the fixed plane. The technique for determining the transformation equations is known as polynomial warping and it employs a bivariant polynomial to effect the transformation.

We have implemented and tested this approach for both the second and third order cases. The second order transformation can be locally quite good but is generally not adequate to superimpose two images at high resolution. The third order algorithm with some control point choices gave almost perfect super-

positions. A different selection of valid register points can give results that are even worse than the second order case. We do not at present know the origin of this difficulty. In the initial phases of the production work it is likely that we will have to use significant operator intervention with these transformation routines and perhaps do the transformation in a series of local regions. This will be greatly facilitated by the display capabilities we are developing as part of the data base (section D)

C. Marker Oligonucleotides:

One of our goals was to accomplish rectification of images from rather distantly related strains. We had hoped that a standard set of marker oligonucleotides could be developed to accomplish this, preferably in a double label format where the marker would only be present on one of two images and thus would not obscure information. The CDC group has worked very hard on this problem, and we have had frequent interactions regarding this. It is now possible to make markers, and to predict closely where they will go. The double labeling idea has proven to be very untenable, and as a result it is necessary that the marker to RNA ratio must be tightly controlled if the markers are to be seen without obscuring real spots. This too is not possible without over complicating the experimental procedures.

We nevertheless believe that we can stil accomplish our goal by a prototype pattern approach. As the data accumulates various obvious groups become apparent. Once a significant number of any type are encountered one isolate can be selected as a prototype pattern. A subset of a standard marker set (i.e. we will exclude any marker that will be obscuring in that particular strain) will

be included with a second batch of the RNA and a new fingerprint produced. This will allow matching with other prototype patterns. Patterns within the group will be easily matched to the prototype without markers due to the very high similarity. The presence of markers on the prototype patterns will also allow a more uniform definition of the spots to be considered during any individual binary comparison. It would also be very useful if the CDC group were able to supply sequence data for some or all of the spots on the prototype pattern. Indeed the availability of complete data of this sort would alleviate much if not all of the need for markers.

D. Data Base:

Recently we have begun construction of the data base itself. The immediate concern is of course storage space. How can one maintain a large number of 640 x 480 pixel images on line without overwhelming the storage capabilities of even a large minicomputer such as the VAX 11/780. The answer is of course that you can't. Instead our goal is to generate a high quality facsimile of each fingerprint. By plotting the boundary pixels of each spot as determined during the segmentation we are able to produce a good likeness of the original image. The quality of this image can be further enhanced by shading the interior of each spot according to its calculated average optical density.

At first sight it would appear that even the storage of a set of coordinates for each boundary pixel would soon become a problem. However if one knows the location of an initial point on the boundary the next point can be found without resort to knowledge of its coordinates by simply specifying which of seven possible directions it is relative to the previous point. This works

because by definition the boundary pixels immediately adjoin one another and so each one must always be one pixel away from its predecessor in some direction. This approach will require preprocessing of the coordinate data to define the direction. The net effect is that a small amount of calculational time will be exchanged for much more efficient storage of the data in the data base. This efficiency of storage will be further augmented by storing two such directions in each computer word. The net effect is an additional 70% reduction in the required storage space. We now estimate that 20-25 facsimile images will require one megabyte of storage. On a dedicated VAX system with a 460 megabyte hard disk almost 9000 images could be handled. In our shared VAX environment however storage will quickly become a problem, and we may only be able to maintain a prototype data base on line unless this problem is resolved.

We have succeeded in displaying such facsimile images, and we have implemented the storage efficiencies described above. In addition we have developed prototypes for several useful display functions. For example windowing on our Lundy T-5684 graphics terminal will allow simultaneous display of six 1/4 resolution images and eventually pairs of images as compared by the transformation algorithm. Indeed we expect to be able to move one image relative to another following the transformation to allow the local refinement in registration that may be needed to facilitate decision making.

With the availability of the facsimile images we have also begun designing the retrieval portion of the data base. The important consideration here is efficiency in the recovery of any desired data. Our intention is to begin with a hierarchical system as it is likely in the present application that data from

sets of closely related strains will frequently be simultaneously retrieved. We also intend to begin exploring the extent to which graphics capabilities can be provided to a remote user of the data base through a personal computer. A DEC Rainbow 100+ is available to us, and thus will be used for this purpose. If this proves feasible we might at a later time attempt to extend support to other models.

E. Data Analysis:

It will be essential to prepare a variety of application programs to interact with the data base. We have an existing 16S rRNA oligonucleotide catalog data base (16) which can be used to maintain any oligonucleotide sequence data the CDC group generates. More importantly this existing package contains several programs that can be readily transferred to the VAX for use in analysis of the image data. Especially pertinent here is an average linkage clustering program that can be used to produce dendrograms and an associated program which will highlight lines on a dendrogram to readily display those which carry a particular attribute such as the presence of a particular spot or a common region of origin.

CONCLUSIONS AND RECOMMENDATIONS:

After one year very significant progress has been made. Essentially all the components of the required software system are now available. In several places such as registration of images the approaches are still a bit crude. In other areas such as segmentation what remains to be done is largely refinement though considerable effort will be required to accomplish the needed improvements. The not inconsiderable remaining task is to integrate these pieces

together. In order to accomplish this we believe the best approach is to enter the fray and see which problems really are the most central. We have thus agreed with the CDC group that they should discontinue most of their developmental efforts and begin full scale production of fingerprints.

Independent of our remaining developmental work we see several problems on the horizon. Most pressing is the need to speed up the segmentation procedure. This can be accomplished very effectively by upgrading the present PDP 11/23 system to the new PDP 11/73 configuration which will give almost a four-fold improvement in processing power at a very reasonable cost.

Further down the horizon we see increasing difficulty in obtaining adequate access to the campus VAX 11/780. Although we have found a very efficient method for storing facsimile fingerprints it is inevitable that we will exceed the relatively meager alotment of on line storage that the University will provide free. In the long run we will have to either pay for the space on a monthly basis or purchase an additional disk drive for the system. Even more threatening is the matter of processor time during class periods. Due to recent internal decisions to establish a "computer intensive environment" on this campus the educational usage of the VAX systems has gone way up. We anticipate that in order to effectively support the significant increase in demand on system resources brought on by the production phase of the project we will require access to a small but dedicated VAX system by the middle second or early third year.

With the onset of the production phase of the work we would strongly recommend that a stronger tie be established between those who wish to use the data

base and those that are developing it. Our group lacks real expertise with the epidemiological aspects of the problem and would profit greatly by input from test users of the system regarding additional useful features and improvements in ease of use. In addition such an interface would greatly facilitate development of documentation. We feel that this can be effectively achieved by equipping the CDC group with an appropriate graphics device for accessing the image data base.

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